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Award Number: W81XWH-09-1-0137

TITLE: Protein Phosphatase 2A signaling in human prostate cancer

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REPORT DATE: June 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
June 2012	Annual	7 May 2011 - 6 May 2012

4. TITLE AND SUBTITLE

Protein Phosphatase 2A signaling in human prostate cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0137

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of South Alabama (USA),
Mobile, AL 36688

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Advanced prostate cancers (PCa) treated with first line androgen-deprivation therapy (ADT) eventually relapse in a hormone refractory or castration-resistant (CR) form. Relapsed disease is highly aggressive and poses an increased risk of morbidity and death. Previously, we demonstrated that *PPP2CA*, which encodes the catalytic-subunit (alpha-isoform) of the protein phosphatase 2A (PP2ACα), is downregulated in CR PCa. The level of PP2ACα was decreased in majority of CR PCa cell lines and cancer lesions as compared to the adjacent normal/benign tumor tissues. Under this project, we have utilized multiple approaches to demonstrate a functional role of PP2A in human prostate cancer progression. Specifically, we have generated and characterized stable *PPP2CA* overexpression (C4-2 and PC3) and knockdown (LNCaP) transfectants and obtained experimental evidence (*in vitro*) for the role of PP2A downregulation in growth, androgen depletion- resistance and aggressive behavior of prostate cancer cells. We have also developed *in vivo* experimental support for a suppressor role of PP2A in prostate cancer progression using orthotopic mouse model. Our data strongly suggest that downregulation of PP2A is associated with human prostate cancer progression and restoration of PP2A activity may be an effective approach for the treatment of the advanced disease.

15. SUBJECT TERMS
PPP2CA, protein phosphatase 2A, castration-resistance, malignant behavior, prostate cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER**
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INTRODUCTION:

First line of therapy for advanced prostate cancer (PCa) is androgen-deprivation therapy (ADT) through surgical or chemical castration; however, in majority of cases, tumors relapse in a hormone refractory or castration-resistant (CR) form (1). Once the PCa has recurred in CR form, it progresses to a highly aggressive disease with frequent metastasis and poses an increased risk of morbidity and death (1). Previously, we demonstrated that *PPP2CA*, which encodes the catalytic-subunit (alpha-isoform) of the protein phosphatase 2A (PP2ACα), is downregulated in CR PCa (2). The level of PP2ACα was decreased in majority of CR PCa cell lines and cancer lesions as compared to the adjacent normal/benign tumor tissues (2). Another study also reported the downregulated expression of β-isoform of PP2A catalytic subunit ($PP2A_{C\beta}$) in PCa (3). $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ share 97% identity and are ubiquitously expressed; however, $PP2A_{C\alpha}$ is about 10 times more abundant than $PP2A_{C\beta}$ (4). $PP2A_{C\alpha/\beta}$ is a well conserved subunit of PP2A serine/threonine phosphatases, and the *in vivo* activity of PP2A is provided by related complexes that exist either as hetero-dimers or hetero-trimers with scaffold (A) and regulatory (B) subunits (5).

Based on these supporting data, we hypothesized that *dysregulation of PP2A plays an important role in the progression of prostate cancer*.

To test our hypothesis, we proposed three specific aims:

Aim 1: Examine the biological role of PP2Ac in androgen-independent growth and malignant properties of the prostate cancer cells.

Aim 2: Define the molecular pathways that are responsive for the changes in PP2A signaling and establish their association with observed phenotype.

Aim 3: Establish the clinical significance of the experimental findings.

BODY:

Task 1: To develop stable transfectants from the prostate cancer cell lines with knockdown or exogenous expression of PP2Acα.

We reported generation of stable *PPP2CA* overexpression transfectants of castration-resistant C4-2 and PC-3 cell lines in previous year's annual report. We have now generated a *PPP2CA* knockdown LNCaP subline (LNCaP-shPPP2CA) (from pooled *PPP2CA*-knockdown clones) along with its control transfectant (LNCaP-Scr). These cells have been characterized for *PPP2CA* (PP2Acα) expression and activity by immunoblot and malachite green based assay, respectively. We observe that LNCaP-shPPP2CA cells have low PP2ACα expression **(Figure 1A)** and activity **(Figure 1B)** as compared to control (LNCaP-Scr) cells.

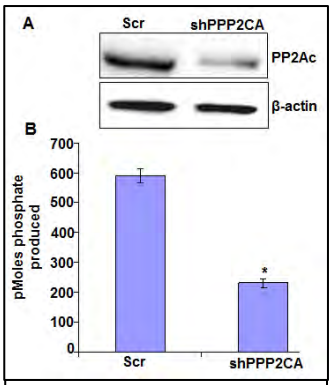

Fig. 1: Stable silencing of *PPP2CA* in LNCaP cells. (A) Immunoblot analysis, **(B)** Assay of PP2A activity. Data shown as mean ± S.D. (n=3); *, p< 0.05.

Task 2: To examine the effect of *PPP2CA* overexpression /silencing on prostate cancer cell phenotype.

We reported the effect of *PPP2CA* downregulation on hormone-refractory growth of PCa cells in previous year's annual report. Now, we have phenotypically characterized stable PCa sublines that either overexpress (C4-2 and PC-3) or are silenced (LNCaP) for *PPP2CA* expression. For growth kinetics, cells ($1x10^4$) were seeded in 6-well plates and growth was monitored by counting the cell number up to 8 days. Our data demonstrate that over-expression of *PPP2CA* in C4-2 and PC3 cells significantly decrease their growth rate, whereas *PPP2CA*-silenced LNCaP cells exhibit increased growth as compared to their respective controls **(Figure 2A)**. The total number of LNCaP-shPPP2CA cells on 8th day of culture indicate 31.6% increase in growth as compared to LNCaP-Scr cells, whereas 34.1% and 35.2% decrease is observed in the *PPP2CA*-overexpressing cells (C4-2-PPP2CA and PC3-PPP2CA, respectively) relative to their respective controls **(Figure 2A)**. Growth analyzed during exponential phase suggest a decrease in population doubling time of LNCaP-shPPP2CA (35.2 h) cells as compared with LNCaP-Scr (48.1 h) cells, whereas C4-2-PPP2CA and PC3-PPP2CA cells exhibit an increase in doubling time (34.7 and 38.9 h, respectively) compared with controls [C4-2-Neo (27.2 h) and PC3-Neo (29.1 h)] cells, respectively **(Figure 2B)**. Altogether, our findings demonstrate that PP2A-downregulation potentiates growth of prostate cancer cells.

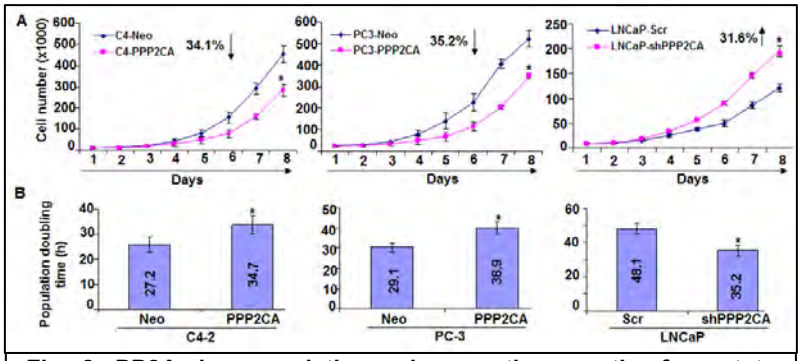

Fig. 2: PP2A downregulation enhances the growth of prostate cancer cells. (A) Cells ($1x10^4$) were seeded in 6-well plates and growth was monitored by counting the cell number upto 8 days. **(B)** Doubling time (h) was calculated during exponential phase. Data shown as mean ± S.D. (n=3); *, p< 0.05.

In last year's annual report, We presented our data on the effect of PP2A downregulation (by transient silencing or pharmological inhibition) in androgen-depletion resistance of LNCaP prostate cancer cells. We have now examined the effect of *PPP2CA*-overexpression on the growth of C4-2 and PC3 cells under androgen-depleted condition. For this, we performed plating efficiency assay, an ideal test to monitor growth in long-term, under steroid-supplemented and –reduced conditions. Cells were seeded at low density ($1x10^3$ cells/well) in steroid-supplemented (FBS) and -reduced (CSS) media. After 2 weeks, colonies were stained with crystal violet, visualized, photographed, and counted using Image analysis software (Gene Tools, Syngene, Frederick, MD). Our data demonstrate that plating efficiency of *PPP2CA*-overexpressing C4-2 and PC-3 cells is decreased (53.4% and 43.3%, respectively), as

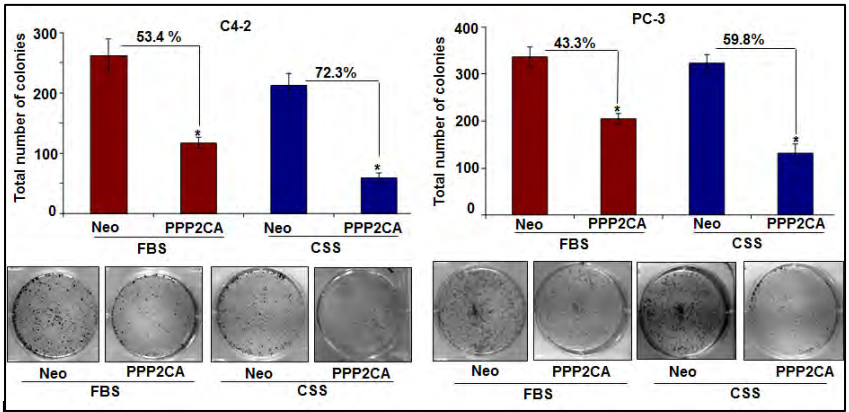

Fig. 3. Upregulation of PP2A suppresses androgen-depletion resistance of prostate cancer cells. Cells were seeded at low density ($1x10^3$ cells/well) in steroid-supplemented (FBS) and -reduced (CSS) media. After 2 weeks, colonies were stained with crystal violet, and visualized and photographed using imaging system. Bars represent mean ± S.D; n=3; *, p< 0.05.

compared to their respective controls under steroid-supplemented condition **(Figure 3)**. Interestingly, plating efficiency is decreased further (~72.3% and 59.8% in C4-2-PPP2CA and PC-3-PPP2CA, respectively) under steroid-deprived condition **(Figure 3)**. Thus, our data provide additional *in vitro* support for an inhibitory role of PP2A in castration-resistant growth of prostate cancer cells.

Since castration-resistant stage of PCa is associated with increased aggressiveness (6), we next investigated the association of PP2A downregulation with malignant behavior of prostate cancer cells. We first examined the effect of PP2A activity modulation on cell migration (by trans-well chamber assays) and invasion (migration through a Matrigel-coated porous membrane) as previously described (7). Data show that number of migrating cells are decreased in *PPP2CA*-overexpressing C4-2 (2.3 fold) and PC-3 (2.2 fold) cells as compared to their respective controls, whereas a 2.4 fold increase is observed in *PPP2CA*-knockdown LNCaP cells **(Figure 4)**. Similarly, we observe a decrease in invasiveness of *PPP2CA* overexpressing C4-2 (2.7 fold) and PC-3 (2.8 fold) cells as compared to their respective control cells, whereas it is increased (3.0 fold) in *PPP2CA* silenced LNCaP cells (**Figure 4**). Another behavioral property associated with tumor cells is decreased cell-cell adhesion that is required to facilitate its dissemination. Therefore, we next examined the effect of *PPP2CA*-overexpression on homotypic interaction of prostate cancer cells in a cell aggregation assay. Our data show an increased cell-cell interaction in *PPP2CA* overexpressing C4-2 and PC-3 cells as compared to their respective controls **(Figure 5)**. Likewise, we also observe decreased cell-cell interaction in *PPP2CA* silenced LNCaP cells as compared to the control cells **(Figure 5)**. Altogether, our data indicate that PP2A downregulation is associated with aggressive behavior of the prostate cancer cells.

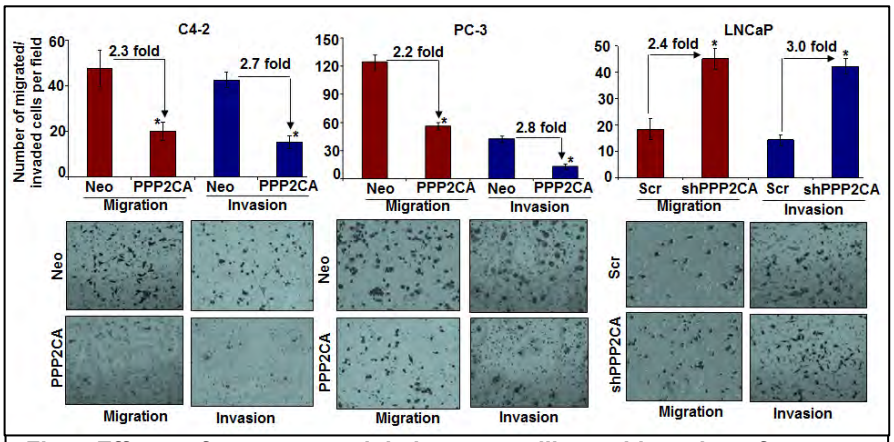

Fig 4. Effects of *PPP2CA* modulation on motility and invasion of prostate cancer cells: Cells were seeded in a transwell chamber (8µ pore size, non-coated or Matrigel-coated) and allowed to migrate or invade through Matrigel under chemotactic drive for overnight. Next day, the cells that did not migrate or invade through were removed and migrated cells were stained using a commercial kit. Images were taken in 10 random fields (magnificationx100) and cell number counted. The data is presented as the mean ± S.D, (n=3). *, p<0.05.

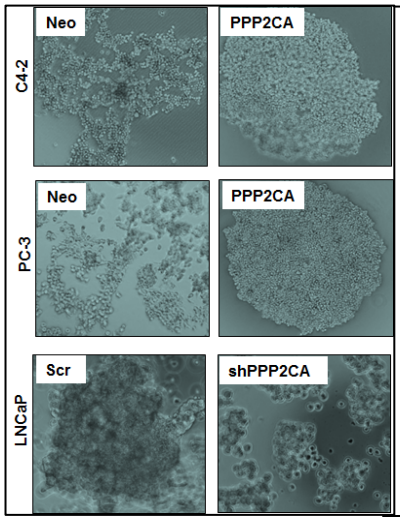

Fig. 5. Effect of PP2A modulation of cell-cell interaction: Cells ($20x10^3$) in a 20µL media were dropped on to inner surface of Petri-dish lid and allowed to hang overnight. Next morning, lid was open and cells were observed under micro-scope and picture taken (magnification x 100). Data show that less cell-cell interaction in PP2Ac-down-regulating PCa cells as compared to their respective

Cancer cells lose their epithelial characteristics and gain a more mesenchymal phenotype as they progress, a process referred to as epithelial to mesenchymal transition (EMT) (8). As mesenchymal cells are relatively more motile and exhibit less cell-cell communication, we investigated a role of PP2A in EMT of PCa cells. For this, we examined the expression of protein markers associated with epithelial (E-cadherin) and mesenchymal (N-cadherin, Vimentin, Slug, Snail and Twist) phenotypes of a cell by immunoblot analysis. Our data show an increased expression of epithelial and decreased expression of mesenchymal markers (except Snail) in *PPP2CA*-overexpressing C4-2 and PC-3 cells as compared to respective controls or vice versa in *PPP2CA*-silenced LNCaP cells **(Figure 6)**.

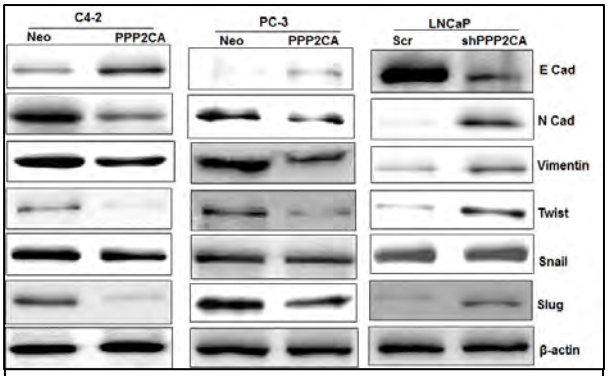

Fig. 6: Effects of PP2 A modulation on the expression of EMT markers. Expression of various epithelial (E-cadherin) and mesenchymal (N-cadherin, Vimentin, Slug, Snail and Twist) markers were examined by immunoblot analyses.

In next set of experiments, we examined the role of PP2A downregulation on the tumorigenesis and metastatic property of prostate cancer cells in an orthotopic mouse model of prostate cancer. Immunodeficient male mice (4 to 6-week old) were purchased from Harlan Laboratories (Prattville, AL) *PPP2CA* overexpressing (PC3-PPP2CA) or control (PC3-Neo) cells were harvested from sub-confluent culture and number of viable cells were counted by dye exclusion assay. Cells were suspended in HBSS medium at a concentration of 10^6 viable cells per 50 µl. Mice were anesthetized with intraperitoneal injection of ketamine and xylazine mixture (4:1), and their abdomen cleaned. A small midline incision was made to expose the prostate gland and cells ($1x10^6$) were injected into the dorsal prostatic lobe using a 27-guage needle. The abdominal wound was closed in two layers and animals were monitored every alternate day. At the end point (30 days post-implantation), mice were sacrificed by CO_2 asphyxiation and autopsied. Prostate tumors were resected, weighed and measured for their dimensions using vernier calipers. Tumor volume was calculated by the following formula: $(AxB^2)/2$, where A is the larger and B is the smaller of the two dimensions. Visible metastases in the regional and distant lymph nodes, lung, liver and spleen as well as other organs were recorded and metastatic tissues collected and formalin-fixed for analysis. Our data demonstrated tumor incidence in all the mice of both the groups, however, mice injected with PC-3-PPP2CA cells had relatively smaller tumor as

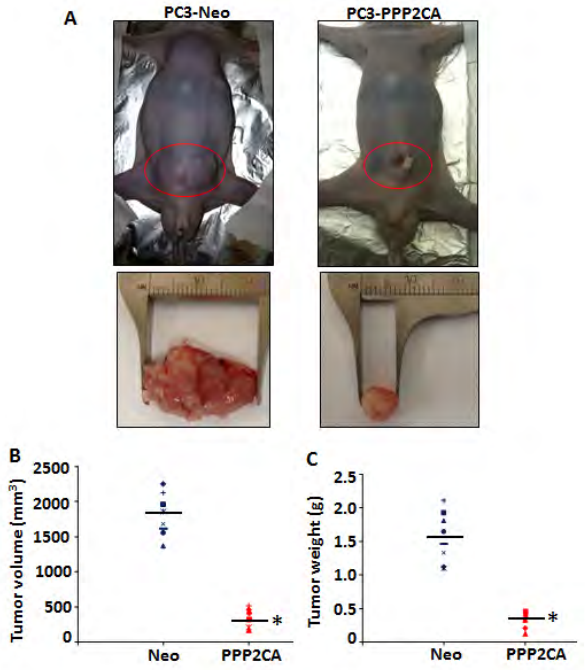

Fig. 7: *PPP2CA* overexpression decreases tumorigenicity of prostate cancer cells. PC3 cells (*PPP2CA*-overexpressing and control) were injected in mouse prostate and their growth in vivo was monitored. **(A)** Mouse carrying tumors generated from control (PC3-Neo) or *PPP2CA*-overexpressing PC3 cells (upper panel) and representative excised tumors (lower panel). **(B)** Average volume and **(C)** weight of the tumors from control and PP2A overexpressing group. Solid lines indicate the mean values (n=8). *, $p<0.05$.

compared to those injected with the control (PC3-Neo) cells **(Figure 7A)**. Average volume and weight of tumors in PC3-PPP2CA group were 317.1 mm^3 (range from 171.5 to 490.8 mm^3) and 0.31 g (range from 0.13 to 0.46 g), respectively as compared to 1803.98 mm^3 (range from 1369.9 to 2254.0 mm^3) and 1.56 g; range from 1.1 to 2.11 g in PC-3–Neo group **(Figure 7B and C)**. Altogether, our data provide strong evidence a role of PP2A downregulation in the progression and metastasis of prostate cancer.

Task 3: To investigate the effect of PP2A on androgen receptor (AR)-dependent and –independent signaling pathways.

We reported studies proposed under this task in our last year's annual report, where we utilized transient gene silencing and specific pharmacological inhibitors to dissect the mechanistic routes downstream of PP2A. To validate the effect of PP2A modulation in our stable transfectants, we examined the activation of ERK and Akt by immunoblot analyses using specific antibodies. As expected, our sata showed a decreased phosphorylation of Akt and ERK in *PPP2CA*-overexpressing C4-2 and PC-3 cells as compared to their respective controls or vice versa in *PPP2CA* silenced LNCaP cells **(Figure 8)**.

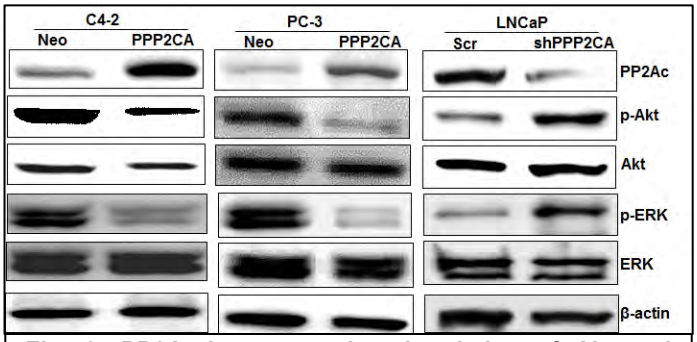

Fig. 8: PP2A decreases phosphorylation of Akt and ERK. Total protein was isolated and effect of PP2A modulation on Akt and ERK activation was examined by immunoblot assay. β-actin was used as internal control.

Task 4: To examine the expression, localization and/or activation profiles of PP2Ac, AR, Akt and ERK in human prostate cancer.

We have been procuring prostate cancer clinical specimens, and are in the process of standardizing immunohistochemical assays.

KEY RESEARCH ACCOMPLISHMENTS:

- We have generated and characterized stable *PPP2CA* overexpression (C4-2 and PC3) and knockdown (LNCaP) transfectants.
- We have obtained experimental evidence (*in vitro*) for the role of PP2A downregulation in growth, androgen depletion- resistance and aggressive behavior of prostate cancer cells.
- We have also developed *in vivo* experimental support for a suppressor role of PP2A in prostate cancer progression using orthotopic mouse model.

REPORTABLE OUTCOMES (during this funding period)

We presented a poster entitled "Protein phosphatase 2A (PP2A) downregulation is associated with aggressive and castration-resistant phenotypes in prostate cancer" by Bhardwaj A, Srivastava SK, Singh S, Arora A, Honkanen RE, Grizzle WE, Reed E and Singh AP, in 103rd Annual Meeting of American Association for Cancer Research (AACR), held at Chicago, Illinois, March 31-April 4. (Manuscript under preparation).

CONCLUSION

Downregulation of PP2A is associated with human prostate cancer progression suggesting that a therapeutic approach that enables restoration of PP2A activity may be effective for treatment of the advanced disease.

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APPENDIX

Bhardwaj A, Srivastava SK, Singh S, Arora A, Honkanen RE, Grizzle WE, Reed E and **Singh AP**. Protein phosphatase 2A (PP2A) downregulation is associated with aggressive and castration-resistant phenotypes in prostate cancer. Poster presented in 103rd Annual Meeting of American Association for Cancer Research (AACR), held at Chicago, Illinois, March 31-April 4.

Protein phosphatase 2A (PP2A) downregulation is associated with aggressive and castration-resistant phenotypes in prostate cancer

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Abstract # 3991

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous malignancy and the second leading cause of cancer related death in males in the United States (1). First line of therapy for this advanced disease is androgen-deprivation therapy (ADT) through surgical or chemical castration; however, in majority of cases, tumors relapse in a hormone refractory or castration-resistant (CR) form (2). Once the PCa has recurred in CR form, it progresses to a highly aggressive disease with frequent metastasis and poses an increased risk of morbidity and death (2). Previously, we demonstrated that *PPP2CA*, which encodes the catalytic-subunit (alpha-isoform) of the protein phosphatase 2A ($PP2A_{C\alpha}$), is downregulated in CR PCa. The level of $PP2A_{C\alpha}$ was decreased in majority of CR PCa cell lines and cancer lesions as compared to the adjacent normal/benign tumor tissues (3). Recently, we have also shown that PP2A downregulation sustains growth of PCa cells under steroid-deprived conditions through a novel mechanism, whereby loss of PP2A-mediated checkpoints leads to the activation of Akt and ERK and partially maintains androgen receptor (AR) signaling (4).

HYPOTHESIS

Downregulation of PP2A promotes castration-resistance and aggressive malignant behavior in prostate cancer cells

METHODOLOGY

For ectopic *PPP2CA* overexpression, castration-resistant (C4-2 and PC-3) PCa cell lines were transfected with pCMV6-*PPP2CA*. Whereas, for the knockdown of *PPP2CA*, castration-sensitive PCa (LNCaP) cell line was transfected with pGFP-V-RS-*shPPP2CA*. Expression and activity of catalytic subunit of PP2A ($PP2A_{C\alpha}$) was determined by immunoblot and melachite green-based enzyme assay kit (Upstate Biotech.), respectively. Growth kinetics was performed by monitoring the cell number at different time intervals. Population doubling time was calculated during exponential growth phase (96–144 h) using the formula: Td = 0.693 t/ln (Nt/N0), where t is time (in h), Nt is the cell number at time t, and N0 is the cell number at initial time. For anchorage–dependant clonogenicity assay, cells were plated in 6-well plate and grown for 2 weeks. Colonies were subsequently stained, photographed and counted. For migration and invasion assays, cells were plated in top chamber of non-coated or Matrigel-coated membranes, respectively, and allowed to migrate or invade overnight. Thereafter, migrated/invaded cells were fixed, stained, photographed and counted in 10 random view fields. For cell aggregation assay, cell suspension were placed onto the inner surface of lid of a Petri dish and hanged overnight to allow cell-cell interaction. Effects of overexpression and knockdown of *PPP2CA* on various proteins were examined by immunoblot analysis.

KEY FINDINGS

- PP2A negatively regulates the activation of Akt and ERK signaling pathways.
- Restoration of PP2A activity suppresses growth and androgen-depletion resistance in CR prostate cancer cells.
- PP2A decreases prostate cancer cell motility and invasiveness, while increases homotypic interactions.
- Downregulation of PP2A is associated with epithelial to mesenchymal transition (EMT) of prostate cancer cells.

Fig. 1: Ectopic expression of *PPP2CA* in castration-resistant prostate cancer cells

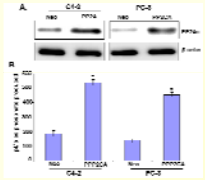

Fig 1. Expression and activity of $PP2A_{C\alpha}$. **(A)** Immunoblot analysis of $PP2A_{C\alpha}$ expression; **(B)** Activity of $PP2A_{C\alpha}$ in stable pooled populations of *PPP2CA*-overexpressing C4-2 and PC-3 (-PPP2CA) and their respective empty vector (-Neo) transfected control cells. Data from triplicate experiments (mean ± S.D) (n=3); *, p<0.05.

Fig. 2: Overexpression of *PPP2CA* negatively regulates Akt and ERK activation

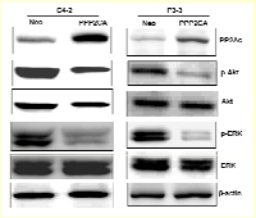

Fig. 2: PP2A decreases phosphorylation of Akt and ERK. Immunoblot analyses was performed for p-Akt/Akt, p-ERK/ERK in *PPP2CA*-overexpressing PCa cells along with their respective controls. β-actin was used as internal control. Data showed that phosphorylation of Akt and ERK was decreased in *PPP2CA* overexpressing cells.

Fig. 3: Upregulation of *PPP2CA* decreases growth of castration-resistant prostate cancer cells

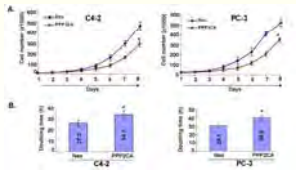

Fig. 3: Association of PP2A with growth characteristics of castration-resistant PCa cells. **(A)** Cells (1x10⁴) were seeded in 6-well plates and growth was monitored by counting the cell number upto 8 days. **(B)** Doubling time (h) was calculated as described in methodology. Data shown as mean ± S.D. (n=3); *, p< 0.05.

Fig. 4: *PPP2CA*-overexpression suppresses clonogenic ability of castration-resistant prostate cancer cells

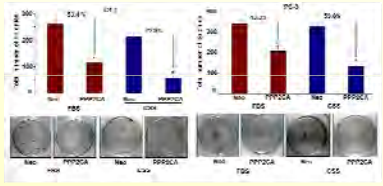

Fig. 4: Clonogenic ability: Cells were seeded at low density (1x10³cells/well) in steroid-supplemented (FBS) and -reduced (CSS) media. After 2 weeks, colonies were stained with crystal violet, and visualized and photographed using imaging system. Bars represent mean ± S.D. n=3; *, p< 0.05.

Fig. 5: *PPP2CA*-overexpression diminishes migration and invasion ability of castration-resistant prostate cancer cells

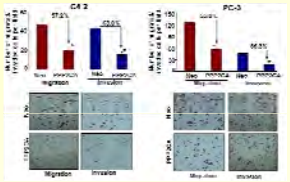

Fig 5. Effects of ***PPP2CA*-overexpression on motility and invasion ability of prostate cancer cells:** Migration and invasion assays were performed as described in methodology. Bars represent the mean ± S.D (n=3) of number of migrated or invaded cells per field; *, p<0.05.

Fig. 6: Overexpression of *PPP2CA* enhances cell-cell interaction

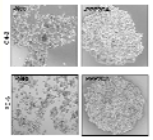

Fig. 6. Cell aggregation assay: Effect of PP2A on cell-cell interaction was determined by hanging drop assay. Data showed that after overexpression of *PPP2CA* cell-cell interaction was increased in both C4-2 and PC-3 cells as compared to their respective controls.

Fig. 7: Overexpression of *PPP2CA* causes reversal of epithelial to mesenchymal transition (EMT) in castration-resistant prostate cancer cells

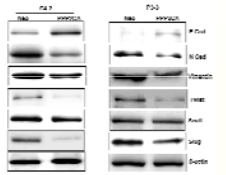

Fig. 7: Effects of ***PPP2CA*-overexpression on the expression of EMT markers.** Expression of various epithelial (E-cadherin) and mesenchymal (N-cadherin, Vimentin, Slug, and Twist) markers were examined by immunoblot analyses. Data showed that *PPP2CA* overexpression was associated with gain of epithelial and loss of mesenchymal markers, indicating its role in MET.

Fig. 8: *PPP2CA-knockdown* promotes growth and malignant properties of castration-sensitive prostate cancer cells

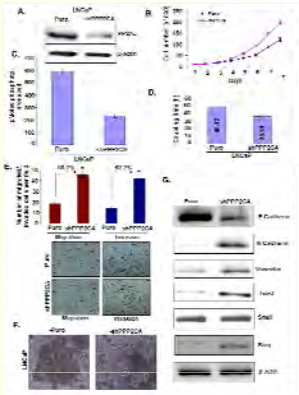

Fig. 8: Effects of *PPP2CA*- silencing on castration-sensitive (LNCaP) prostate cancer cells. Data showed that decreased expression **(A)** and activity of $PP2A_{C\alpha}$ **(B)** were associated with increased growth **(C)**, lower doubling time **(D)**, enhanced motility and invasion **(E)**, diminished cell-cell interaction **(F)**, and loss of epithelial and gain of mesenchymal markers **(G)** in castration-sensitive dependent (LNCaP) prostate cancer cells.

CONCLUSION

Downregulation of PP2A augments castration-resistance and malignant behavior of prostate cancer. Therefore, restoration of PP2A activity may be an alternative therapeutic approach against advanced prostate cancer.

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Funding support ($$$)

Department of Defense/US Army & USAMCI